

Rhythmic Leaf Movements in Biloxi Soybean and Their Relation to Flowering¹

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ABSTRACT

The rhythmic leaf movement of Biloxi soybean (*Glycine max*) and its relationship to the rhythmic flowering response were studied. The movements of fully expanded trifoliate leaves were recorded with kymographs and time lapse photography in growth chambers. A comparison between the leaf movement rhythm and the rhythmic flowering response indicates that a high degree of similarity exists between the two rhythms. A definite relationship was shown to exist between the direction of the leaf movement and the photophil-photophobe phases of the rhythmic flowering response.

Short light perturbations may affect flowering by interacting directly with the flowering process while not affecting the basic endogenous rhythm. Long light perturbations may affect flowering by phase shifting the basic endogenous rhythm. Thus, light perturbations appear to have a dual effect on the flowering response of Biloxi soybean. The hypothesis that both the flowering rhythm and the leaf movement rhythm are coupled to the same basic oscillator is supported by the similarity of the phase shifts induced in the two rhythms by identical light perturbations.

Bünning (3) proposed that the photoperiodic response of flowering may be explained on the basis of a circadian rhythm of sensitivity to light. According to his hypothesis, the 24-hr period of this flowering rhythm is divided into 12-hr segments called the photophil and skotophil phases. For short-day plants, Coulter and Hamner (10) have used the more descriptive term photophobe phase to represent Bünning's skotophil phase. In short-day plants photophil is defined as the phase of the rhythm during which light promotes flower induction, while photophobe is the phase of the rhythm during which light inhibits flower induction. Pittendrigh (21) subscribes, in general, to Bünning's proposition with respect to photoperiodic time measurement, but is more explicit with respect to the interaction of light with the rhythm. He visualizes two distinct functions for light: induction and entrainment. This paper provides supporting evidence for such a dual role of light in photoperiodism.

Bünning further hypothesized that the rhythmic alternation of the two phases is coupled to the same basic physiological clock that controls all circadian rhythms of the plant. Thus, the phase of the light-sensitive rhythms of the flowering response may be indicated by or interpreted from leaf position. According to Bünning (6), the photophil phase would then correspond to the horizontal or day position of the leaf, while the skotophil phase would correspond to the closed vertical or night position of the leaf (Fig. 1). Bünning has used leaf movement data to explain some of the photoperiodic flowering responses of Biloxi soybean. However, we felt that more information was needed concerning the extent of the relationship between these two factors before the study of one may be used to explain the behavior of the other.

The rhythmic flowering of Biloxi soybeans has been demonstrated conclusively with the variable cycle length experiments (1, 18) and with light perturbation experiments (10, 19, 22). With ample documentation of this rhythmic response in Biloxi soybean, it is now profitable to investigate in depth the nature of Biloxi soybean leaf movements. From results of such leaf movement experiments it would be possible to determine whether or not there is a relationship between the flowering response and leaf movement. Thus, evidence might be obtained as to whether or not the leaf movements and the photoperiodic flowering response are controlled by a single basic timing mechanism. Possible further insights into the mechanism of the flowering response might also be gained.

MATERIALS AND METHODS

Biloxi soybean seeds (*Glycine max* L. [Merr.]) used in all experiments were obtained from Dr. H. A. Borthwick. Culturing methods were similar to those previously reported by Shumate *et al.* (22). After the third trifoliate leaf had fully expanded, the plants were moved from the long-day greenhouse to a treatment room having GE Power Groove fluorescent light (1000 ft-c) and maintained at 28 ± 1 C during the light period and 21 ± 1 C during the dark period. In most experiments four plants were used per treatment and the experiments were repeated at least once.

In all of the experiments the plants were given two 24-hr cycles consisting of 16 hr of light and 8 hr of dark (16L:8D) in the treatment rooms just before the experiment. This pretreatment acclimatized the plants to their new environment and reduced the rocking movement of the leaves which occurs as a result of moving the plants. To induce maximal flowering response, all plants were given seven consecutive experimental cycles. In the light perturbation experiments, each experimental cycle consisted of an 8-hr photoperiod followed by 64 hr of dark. The light perturbations were given in each of the seven experimental cycles during the 64-hr dark period.

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The light source for the perturbations was the same as that used during the 8-hr photoperiod. In the variable cycle length experiments, each experimental cycle consisted of an 8-hr photoperiod followed by dark periods of appropriate lengths.

The movements of the third trifoliate leaves were studied. Only fully expanded leaves were used to eliminate possible confusion in the kymograph records caused by the growth of the leaf. Measurement of the leaf movement was done primarily with a kymograph designed in this laboratory. The tip of the mid-leaflet of the trifoliate leaf was connected to the pen arm with a thread. In some experiments leaf movements were recorded with a time lapse camera using high speed infrared film and infrared lamps. The angle between the leaf blade and the stem of the plant was measured with the aid of a modified Boscar film reader. Graphs were then drawn based on the average angle of four leaves.

In order to obtain graphs from the kymograph records representing the average leaf movement for each treatment, a scale with 0 representing the lowest point on the curve (day position) and 10 representing the highest point (night position) was assigned to each kymograph record. The numerical values representing the position of the leaf at 2-hr intervals were recorded for each kymograph record. All of the values obtained for a particular time in each treatment were summed and the average value was calculated. The kymograph data were then inverted in order that an upward movement on the curve would correspond to an upward leaf movement and vice versa. In this way a single average curve was obtained for each treatment.

After the 3-min light perturbation and variable cycle length treatments, the plants used were grown in the long-day greenhouse for at least 6 weeks and dissected to determine their flowering response. These results were compared and combined with results obtained by others (1, 10, 18) for use in comparing the rhythm of the flowering response with the leaf rhythm.

RESULTS

Leaf Movement Response to 24-hr Light-Dark Cycles. It has been previously reported that the leaf movement of Biloxi soybeans can be classified as a circadian rhythm (2, 5). However, a strong photonastic response of Biloxi soybean leaves has also been reported (2, 14). The purpose of this experiment was to determine to what extent the movement of the leaves was correlated with the light-to-dark and dark-to-light transitions in 24-hr cycles. Two groups of five plants were used. The first group received short-day cycles (8L:16D). The second group received long-day cycles (16L:8D).

As seen in Figure 1, the leaves of both groups respond photonastically to the dark-light transition at the beginning of the light period by moving to the horizontal or day position. In both groups the leaves remained in their horizontal position until the light-dark transition which occurs at the end of the light period. At this time they moved down to the vertical night position. Based on the two photoperiods used, it would appear that light has a controlling influence on the leaf movement mechanism. This is not in perfect agreement with Bünning's idea that the position of the leaf may be used to indicate the photophil or skotophil phase of the flowering response (4).

While our results did not agree with Bünning's³ with respect to the leaf movement during the light (5), evidence was seen of the ongoing rhythm during the dark periods of both treatments. The leaves in both cases begin to move toward their day posi-

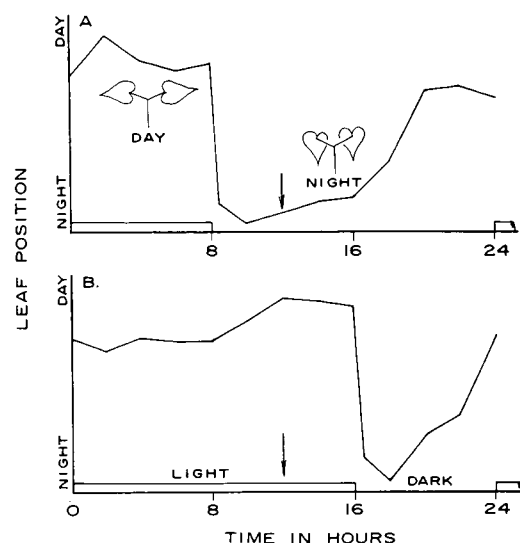


FIG. 1. Comparison of the leaf movement on two different 24-hr cycles. A: 8 hr of light and 16 hr of dark; B: 16 hr of light and 8 hr of dark. The arrows indicate the critical day length for Biloxi soybeans. In this and subsequent figures, the curves represent the mid-leaflet movements of trifoliate leaves.

tions prior to the beginning of the next light period. This anticipation of the coming light period reflects the rhythmic response of the leaf.

Comparison of Leaf Movement and Flowering Rhythm during a 72-hr Cycle. Since rhythmic leaf movements were noted during the dark period in the previous experiment, it was decided to conduct studies using longer dark periods to permit the leaf to express its rhythmic movements. The same 8L:64D cycles as used in the flowering experiments of Coulter and Hamner (10) were used so that a comparison could be made between the flowering rhythm they found and the leaf movement that we might obtain.

In studying the results it was noted that the leaf rhythms during the first three cycles differed from the rhythms on the last four cycles (at least as shown on the kymograph record). When the leaf movement rhythm that we obtained during the first three cycles is compared to the photophil and photophobe phases of the flowering rhythm reported by Coulter and Hamner (10), a correlation is apparent (Fig. 2). The leaf moves downward during the photophil phase and moves upward during the photophobe phase. Thus the correlation is between the direction of leaf movement and phase of the flowering rhythm.

The time lapse photographic records of the fourth to seventh cycles show that when the light period ends the leaf begins what can best be described as a rocking movement (control curves in Fig. 3). This type of movement has also been observed when the plants were transported or were physically disturbed in other ways. It is possible that the rocking movement is a stress reaction which, in this experiment, is induced by the abnormal cycle lengths. In all cases the rocking movement ceases by the 24th hr of the cycle and the circadian rhythm then continues normally. During the first 24 hr this rocking movement obscures the circadian leaf movement on the kymograph record. The rocking movement is not seen during the first two or three cycles.

Leaf Movement as Affected by a 3-min Light Perturbation Given during Photophobe Phases. Shumate *et al.* (22) made a thorough investigation of light perturbation effects during the photophobe phases of a 72-hr cycle on the flowering rhythm in

³ It may be noted that Bünning studied the primary leaf and we studied the trifoliate leaf.

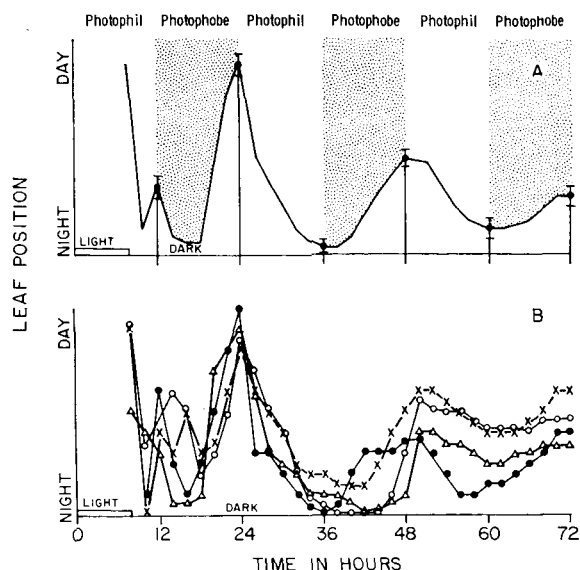


FIG. 2. Comparison of leaf movement rhythm with the phases of the flowering rhythm. A: The leaf rhythm during the 64-hr dark period of a 72-hr cycle is compared with the photophil-photophobe phases of the flowering rhythm from data by Coulter and Hamner (10). The leaf movement curve is the mean of 21 leaves during the first cycle. The vertical bars indicate the standard error at selected points. B: Four individual kymograph records chosen at random from the 21 used in obtaining the average curve in part A.

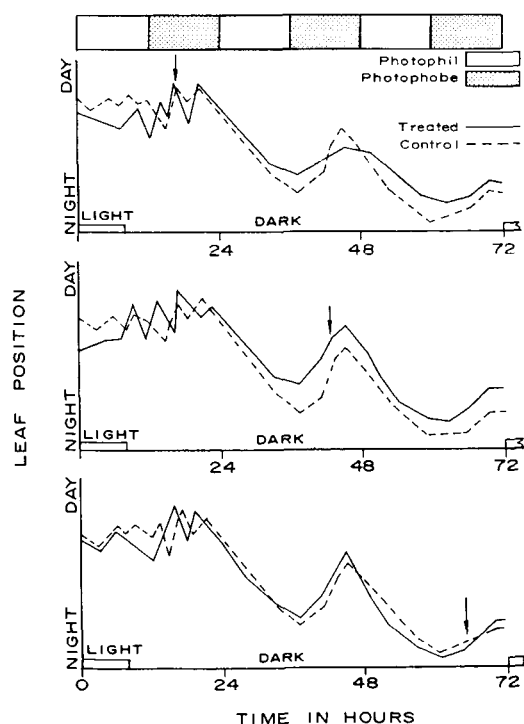


FIG. 3. Effect of 3-min light perturbations on the leaf movement rhythm. The curves represent the leaf movements during the seventh repetition of a 72-hr cycle. Perturbations, indicated by arrows, were given at the times of maximal floral inhibition. Shaded areas represent photophobe phases from data by Shumate and Hamner (22). The rocking movement of the leaves is indicated by the up and down fluctuations of the curves during the first 16 hr of the dark period.

Biloxi soybeans. They found that light perturbations of 3-min duration maximally inhibited flower induction when given at the 16-, 42-, and 66-hr points of the 72-hr cycle (8L:64D). The same light perturbation treatments were given to groups of five plants each in this experiment. Controls were given seven 72-hr cycles without light perturbations. The 3-min light perturbations given at times of maximal floral inhibition have no noticeable effect on the leaf movement rhythm. Data obtained during the seventh cycle of treatment are presented (Fig. 3). The flowering response of the plants, however, was similar to that reported by Shumate *et al.* (22), indicating that 3-min perturbations affect the flowering response but do not apparently affect the leaf movement rhythm.

Light Perturbations of Various Durations Given at 16-hr Point of a 72-hr Cycle. In the previous experiment it was found that 3-min light perturbations had essentially no effect on leaf movement rhythms. However, light perturbations given during long dark periods have been shown to induce phase shifts in many different circadian rhythms (11, 16, 19, 20, 26). To determine whether longer perturbations are necessary to cause a phase shift in the leaf movement rhythms, light perturbations of 30 min, 1, 2, and 4 hr were given to Biloxi soybeans. These light perturbations were all initiated at the 16-hr point of the 72-hr cycle. Light perturbations given at this time are maximally inhibitive to flowering (8, 9). One group received no light perturbation and served as a control. The 30-min and 1-hr light perturbations did not induce a phase shift, and for this reason data are not presented. On the other hand, the 2-hr light perturbation induced a slight phase shift in the rhythm (Fig. 4). In all treatments the light perturbations induced a photonastic response. Thus, while the flowering response at the 16-hr point was sensitive to light perturbations as short as 3 min, the leaf movement rhythm was surprisingly insensitive and was not affected until the duration of the light perturbation was at least 2 hr.

Comparison of Effects of 30-min Light Perturbations Given at Various Times during a 72-hr Cycle. Nanda and Hamner (19), using the variable cycle length method, tested the effect of a 30-min light perturbation given at various times in the cycle on the flowering response of Biloxi soybeans. Perturbations at the 16-hr point resulted in a complete cessation of flowering for all cycle lengths tested. Light perturbations given at the 40-hr point resulted in a distinct phase shift in the rhythm of the flowering response. Light perturbations given at other times during the cycle had no pronounced effect on the phase of the rhythm.

In our experiment 30-min light perturbations were given at the 16-, 24-, 36-, 40-, and 48-hr points of a 72-hr cycle to de-

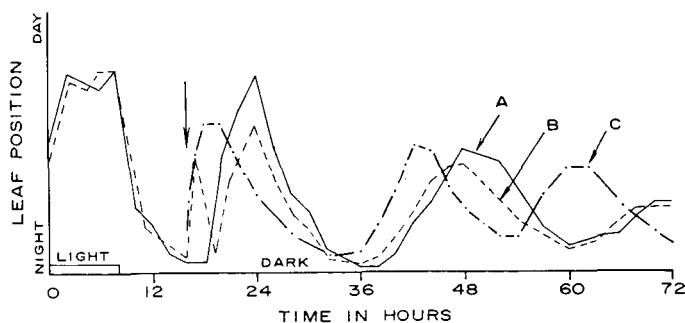


FIG. 4. Effects of 2- and 4-hr light perturbations on the leaf movement rhythm. Perturbations were given at the 16-hr point of a 72-hr cycle. Curves represent the leaf movements of the first exposure to 72-hr cycles. A: Control, no light perturbation; B: 2-hr light perturbation; C: 4-hr light perturbation.

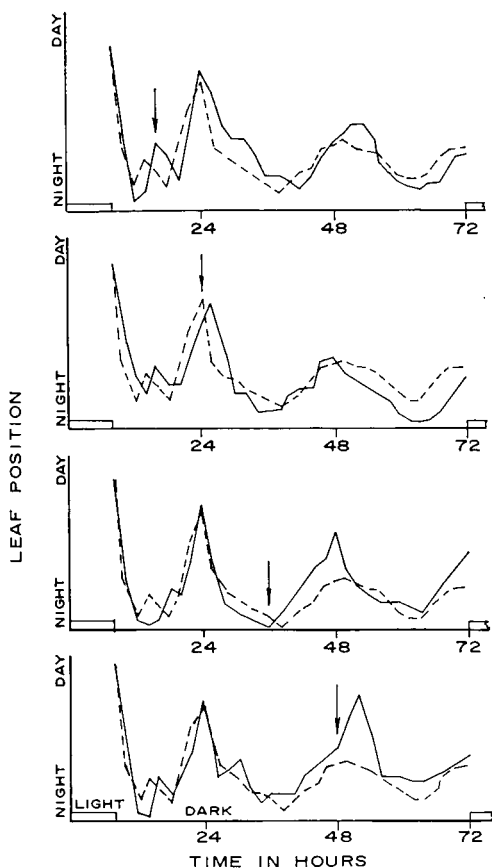


FIG. 5. Effects of 30-min light perturbations on the leaf movement rhythm. Perturbations were given at the 16-, 24-, 36-, and 48-hr points of a 72-hr cycle. (Effects of the perturbation at the 40-hr point is given in Fig. 6A.) Arrow on each curve indicates the time at which the light perturbation was given. Leaf movement curve is the mean of eight leaves during the first cycle. —: Control, no light perturbation; - - : light perturbation curve.

termine their effects on the leaf movement rhythm. Light perturbations given at the 16-, 24-, 36-, and 48-hr points (Fig. 5) had no pronounced effect on the phase of the leaf movement rhythm. The light perturbation at the 16-hr point that resulted in the complete damping of the flowering response (19) produced only a slight photonastic response in the leaf movement, after which the rhythmic leaf movement continued unchanged. On the other hand, the light perturbation at the 40-hr point resulted in a 12-hr phase shift in the leaf movement rhythm (Fig. 6A). This phase shift became most apparent from around the 60-hr point to the end of the cycle. This phase shift was similar to the one that was induced by Nanda and Hamner (19) in the response rhythm of flowering in Biloxi soybeans (Fig. 6B). These results indicate that the leaf movement rhythm and the rhythmic response in photoperiodic flowering may be coupled to the same endogenous oscillator.

Leaf Movement Response in Light-Dark Cycles of Various Lengths. Bünning's hypothesis states that the phase of the rhythm when light is given is paramount to the flowering response. When light-dark cycles having 8 hr of light followed by dark periods of varying duration were given to Biloxi soybeans, the cycle lengths totaling 24, 48, and 72 hr are most effective in inducing flowers, presumably because each 8-hr light period occurs during a photophil phase of the endogenous rhythm while those totaling 16, 32, and 60 hr are the least effective, presumably because each 8-hr light period occurs

during a photophobe phase (18). From this we hypothesized that if the leaf rhythm is closely coupled to the flowering rhythm, the direction of the leaf movement even during a variable cycle length experiment should correlate with the phase of the flowering rhythm.

To test our hypothesis, cycle lengths totaling 24, 32, 48, 60, and 72 hr were given to different groups of plants. Each cycle length was repeated seven times, and the flowering responses and leaf movements of the plants were determined. A relationship was found in each treatment between flower induction and the direction of the leaf movement at the very end of the cycle (Fig. 7, Table I). In those cycle lengths most effective for flower induction, the onset of the 8-hr photoperiod of each succeeding cycle came shortly after the start of the downward movement of the leaf. Of course, immediately after the light-dark or dark-light transitions there are rapid leaf movements. In those cycle lengths which inhibit flower induction, the 8-hr photoperiod of each succeeding cycle coincides with the start of the upward movement of the leaf. This direct correlation of leaf movement direction with flowering response in these variable cycle length experiments gives further evidence of the very close coupling between the movement of the leaves and the phase of the flowering rhythm in Biloxi soybeans.

DISCUSSION

The rhythm found during the dark period may be an expression of the basic oscillator controlling both flowering and leaf movement. A similar hypothesis has been presented by previous investigators (5, 7, 12, 17, and 25). However, our results give evidence that the phase of the flowering response, either photophobe or photophil, may be determined, not by noting the leaf position as in previous investigations, but most importantly by noting the direction of the leaf movement.

It has long been known that a 3-min light perturbation is sufficient to inhibit flower induction (19, 22) when given at the

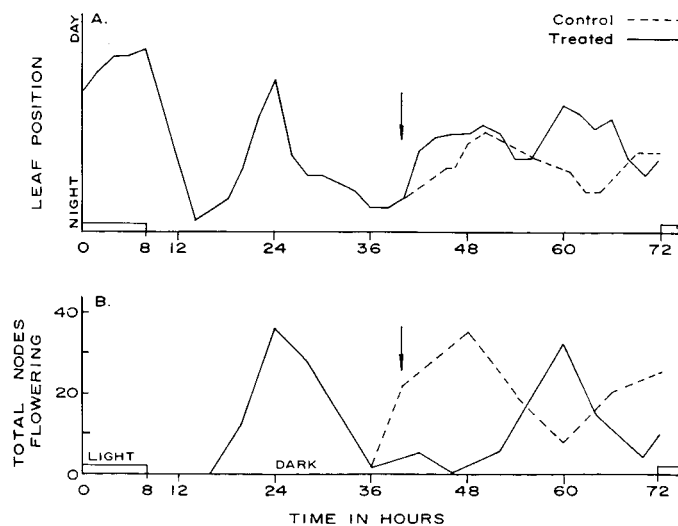


FIG. 6. Comparison of the effects of 30-min light perturbations on leaf movement and the rhythmic flowering response (cf. Fig. 5). A: Leaf rhythm response to a 30-min light perturbation given at the 40-hr point of a 72-hr cycle composed of 8 hr of light and 64 hr of dark. The dashed line represents the control which received no perturbation. The curves are the mean movements of eight leaves during the first cycle. B: Flowering response to a 30-min light perturbation given at the 40-hr point of a 72-hr cycle. The dashed line represents the control which received no light perturbation. Data from Nanda and Hamner (19).

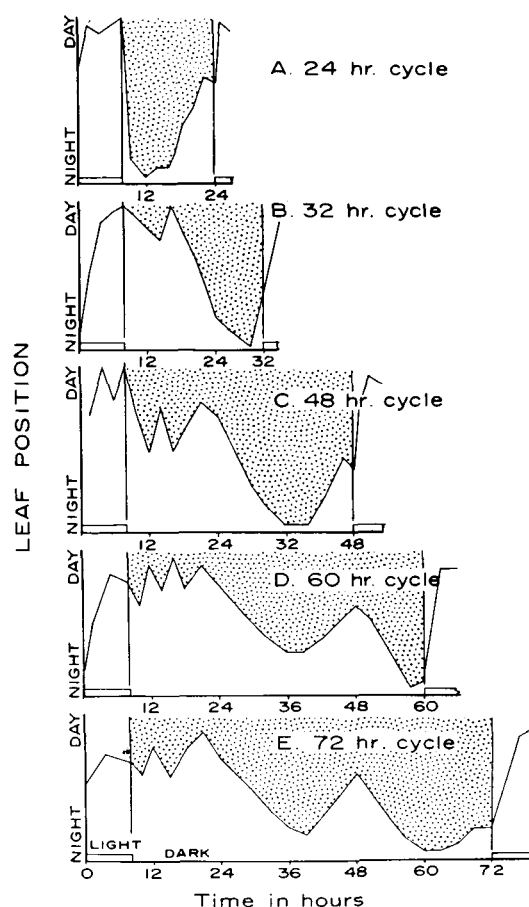


FIG. 7. Leaf movement response to various cycle lengths. The length of the cycles was changed by varying the length of the dark period. Light period was 8 hr long in each treatment. Each curve represents the seventh repetition of that cycle length. The shaded areas represent the dark period in each cycle. Compare this 72-hr cycle (seventh repetition) with Figure 5, where the leaf movement during the first cycle is given.

Table 1. Comparison of Direction of Leaf Movement at Beginning of Light Period of Various Cycle Lengths with Amount of Flowers Induced by Cycle Lengths

Cycle Length hr	Direction of Leaf Movement Prior to Beginning of Light Period	No. of Flower Nodes Induced
24	Down	32
32	Up	1
48	Down	34
60	Up	8
72	Down	31

16-hr point of a 72-hr cycle. This same treatment, on the other hand, has no effect on the leaf movement rhythm, indicating that the basic oscillator controlling the leaf movement was unaffected (Fig. 3). For this reason, an investigation was made of the effects of various light perturbations at the 16-hr point to determine the length of illumination required to affect the basic oscillator. Somewhat surprisingly, the basic oscillation as inferred from the leaf movement rhythm is quite insensitive to light perturbations at this point, requiring at least 4 hr of light to induce a definite phase shift. However, this energy or dura-

tion difference does not rule out the possibility that flowering and leaf movement rhythms are closely coupled. We may assume that the basic oscillator is not perturbed by the 3-min light perturbations since leaf rhythms are not affected. If this assumption is correct, flowering was inhibited not because the 3-min perturbation caused a phase shift in the basic rhythm but because illumination came during a photophobe phase of the flowering rhythm and had a direct effect on flowering. Further evidence to support these assumptions was obtained in Experiment 5. When 30-min light perturbations were given at the 40-hr point of a 72-hr cycle, the leaf rhythm displayed a phase shift comparable to the shift in the flowering rhythm of Biloxi soybeans given identical treatments (Fig. 6). It can be interpreted from the leaf movement records that by the 40th hr of the cycle, the basic oscillation initiated by the 8-hr photoperiod is attenuated. This attenuation may be seen in the decrease of the amplitude of the leaf movements in our results and in a similar decrease in the amplitude of the flowering response curve as reported by Nanda and Hamner (18). Thus, late in the cycle it is possible that the attenuated basic oscillator can be phaseshifted by a 30-min light perturbation, thereby accounting for the identical phase shift of both the leaf movement and the flowering rhythm.

It has become apparent in analyzing the effects of light perturbations on the basic oscillator that light may play a dual role in its interaction with the flowering response. In the first role, light perturbations of relatively short duration (3 min) if given during the photophobe phase, may in some way directly inhibit flowering while having no effect on the basic oscillator, as evidenced by the leaf movement rhythm (Fig. 3). Further evidence that 3-min light exposures may affect flowering without rephasing the basic rhythm is found in work with *Pharbitis* (24). With that plant, the inhibitory effect on flowering of a brief illumination during a photophobe phase could be overcome by subsequent brief illumination during a photophil phase, indicating that the first illumination had not affected the course of the basic rhythm.

In the second role, light perturbations of longer durations affect flowering by phase-shifting the basic oscillator again as evidenced by the effect on the leaf movement rhythm (Fig. 7). The duration of light needed to affect a phase shift appears also to be dependent on the degree of attenuation of the basic oscillator as evidenced by both the leaf movement and flowering response curves (Fig. 6).

Previous papers from our laboratory discussed the participation of a light-on and a light-off rhythm in the photoperiodic flowering response of *Pharbitis* (23) and in the leaf movements of *Xanthium* (15). It should be noted that the present experiments were not designed to determine the presence or the effect of the light-on and light-off rhythms on the leaf movements or flowering in Biloxi soybeans.

In our discussion, we have been inclined to the hypothesis of a single physiological clock controlling both the photoperiodic flowering response and the leaf movements. It is, of course, possible that there are two separate clocks involved in the two responses we have been studying. In fact, the light-off, light-on rhythm that has been reported in the leaf movements of *Xanthium* (15) and in the flowering response of *Pharbitis* (23) might be an indication of a multiple clock system. Hastings (13) has discussed the possibilities and implications of one clock versus many clocks, and it does not seem worthwhile to detail that discussion. We feel that our evidence favors at least the very close coupling of the basic timing for both leaf movement and flowering response rhythms.

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